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CHAPTER 5

Peptide and Protein Hydrogels

LAWRENCE J. DOOLING AND DAVID A. TIRRELL*

California Institute of Technology, Division of Chemistry and Chemical Engineering, 1200 East California Boulevard, MC 210-41, Pasadena, CA 91125, USA

*Email: tirrell@caltech.edu

5.1 Introduction

Proteins are a fascinating class of macromolecules from both functional and structural perspectives. They catalyze the reactions that sustain life, bind ligands with high affinity and specificity, and mediate interactions among biomolecules in complex cellular milieux. Proteins also assemble into higher-order structures that are responsible for the mechanical integrity of cells and tissues. Their diverse functional and structural properties have made proteins important building blocks in the development of new biomaterials.^{1–4}

Hydrogels are physically or chemically cross-linked polymer networks with high water content.^{5,6} Their formation requires a balance between the forces driving the association of polymer chains and those mediating solvation of the network. Given that proteins have evolved to fold and function in aqueous environments and that many proteins self-assemble into larger structures, they would seem to be ideal candidates for use as hydrogel precursors. Indeed, proteins, and more broadly peptides of all sizes, are widely used for this purpose.

The development of peptide and protein hydrogels has been a cross-disciplinary effort combining the knowledge of protein structure and function from biology with the synthesis and characterization tools of macromolecular

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chemistry and materials science. The primary motivation driving this field is the need for implantable scaffolds for soft-tissue engineering,^{7,8} benign methods for encapsulation of cells and biomolecules,^{9,10} matrices for *in vitro* cell culture,¹¹ and injectable delivery vehicles for therapeutics.^{12,13} Moreover, peptide hydrogels show promise as templates for biomineralization¹⁴ and inorganic nanostructures.¹⁵ The ability of proteins to undergo biochemical and structural changes in response to pH and temperature changes, ligand binding, light, and mechanical force also suggests potential applications for peptide hydrogels as biosensors and stimulus-responsive materials.^{16,17} Finally, peptide hydrogels provide simplified systems for studying and engineering the assembly of biological molecules as well as inspiration for the development of self-assembling synthetic structures.

We identify four classes of peptide hydrogels: (1) hydrogels from self-assembling oligopeptides, (2) hydrogels from recombinant proteins, (3) hydrogels that are hybrid materials combining peptides and proteins with synthetic polymers, and (4) hydrogels from naturally sourced proteins and proteoglycans such as collagen. Despite the importance of this last class of hydrogels in cell culture and tissue engineering applications, there are only limited opportunities for engineering and rational design of its macromolecular components, and detailed characterization can be challenging because of material heterogeneity. Therefore, after a brief background on peptide and protein structure, we will limit our discussion to examples from the first three classes of peptide hydrogels before concluding with future directions and challenges in this field.

5.2 Peptide and Protein Structure

Peptides are polymers formed by the condensation of amino acids. Short polymer chains with degrees of polymerization less than approximately 25 amino acids are generally described as oligopeptides, while longer chains are known as polypeptides. The term protein will be reserved for polypeptides that have been synthesized by ribosomal translation.

5.2.1 Peptide and Protein Synthesis

The most basic level of protein structure is the linear sequence of amino acids, also known as the primary structure. The protein sequence is genetically encoded in DNA, or more specifically in the portion of the DNA that is transcribed into messenger RNA. Protein synthesis occurs on the ribosome, which translates the messenger RNA and catalyzes the formation of amide bonds between amino acids and the growing polypeptide chain. Through genetic engineering, it is possible to produce natural or artificial proteins in a wide variety of host organisms, typically *Escherichia coli* but occasionally yeast and higher eukaryotes. Recombinant protein production yields monodisperse polymers with precise sequence control that is not possible with synthetic

polymerization methods. Oligopeptides and short polypeptides are synthesized *in vitro* using solid-phase peptide synthesis (SPPS), with an upper limit of approximately 50 amino acids.¹⁸ In this method, the peptide chain remains tethered to a resin support as each residue is added by amide coupling of a protected amino acid and its subsequent deprotection. Longer sequences can be synthesized by combining oligopeptides using native chemical ligation.¹⁹

With a few important exceptions,^{20,21} nature incorporates only 20 different amino acids into proteins. These are known as the canonical or natural amino acids. While the number of ways in which these monomers can be combined is nearly infinite, the diversity of the canonical amino acid side chains is somewhat limited from the viewpoint of synthetic chemistry. From the perspective of investigators who wish to design new peptide and protein biomaterials, it is advantageous to augment the canonical amino acids with new monomers that carry more diverse functionality, including reactive groups for bioorthogonal chemistry,^{22,23} halogens,^{24–26} and photo-reactive moieties.²⁷ Furthermore, natural proteins contain exclusively L-amino acids, which can influence protein structure and biomaterial assembly.²⁸ Non-canonical amino acids can be incorporated into synthetic oligopeptides, assuming that their side chains do not interfere with the coupling and deprotection reactions or that suitable protecting groups are available. Numerous strategies have also been described for the co-translational incorporation of non-canonical amino acids in a residue-specific²⁹ or site-specific manner.³⁰

5.2.2 Higher-Order Structure and Hydrogel Assembly

Protein secondary structure describes the local conformation of the peptide backbone. Different conformations arise from the rotational degrees of freedom of the N-C α and C α -C bonds. The most common secondary structures are the α -helix and β -sheet, which are characterized by extensive hydrogen bonding between amine and carbonyl groups. Other secondary structures include turns and loops, β -hairpins, β -spirals, and polyproline helices. As discussed in the next section, regular secondary structure is a common feature in peptide and protein hydrogels and provides a basis for classifying these materials.

Tertiary structure is the spatial arrangement of all of the atoms in a protein. It may also be referred to as the folded state of a protein and is closely related to function. Many proteins also have a quaternary structure that describes the assembly of multiple polypeptide subunits. Typically these subunits associate noncovalently through hydrogen bonding, hydrophobic interactions, and salt bridges. Alternatively they may be bound covalently through disulfide bond formation or enzymatic cross-linking. The intermolecular interactions that are responsible for protein quaternary structure closely resemble those that facilitate the self-assembly of peptide and protein hydrogels.

In addition to classification based on the type of peptide precursor (*i.e.* oligopeptides, recombinant proteins, *etc.*) or secondary structures (*i.e.* β -sheets, α -helices, *etc.*), peptide hydrogels can also be described by the mechanism of

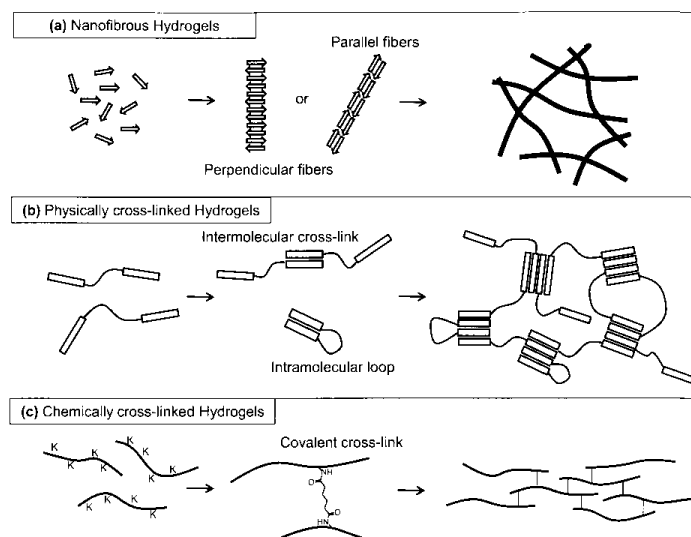


Figure 5.1 Three modes of assembly of peptide hydrogels. (a) Oligopeptide precursors assemble into perpendicular or parallel nanofibers that entangle to form physically cross-linked gels. (b) Artificial proteins and peptide-polymer hybrids contain physical cross-linking domains separated by water-soluble linkers. Noncovalent association of these domains gives rise to intermolecular cross-links and intramolecular loops, which further assemble into hydrogel networks. (c) Hydrogels can also be formed by chemical cross-linking of reactive amino acid side chains such as the ϵ -amine of lysine (K).

network assembly. Figure 5.1 depicts three such mechanisms for forming peptide hydrogels. The first is the gelation of nanofibrous peptide assemblies and is observed predominantly with oligopeptide precursors. Gel formation in such systems involves supramolecular assembly of precursors into nanofibers, -tapes, -tubes, or -ribbons that entangle and form nonspecific physical cross-links. The second mode of assembly is observed in longer multidomain proteins and peptide-polymer hybrids. Network assembly results from the noncovalent association of physical cross-linking domains that are separated by water-soluble linker regions. The final assembly mode is chemical cross-linking and involves covalent bond formation at reactive amino acid side chains such as lysine and cysteine. It should be noted, however, that these assembly mechanisms are not mutually exclusive. For example, nanofibrous hydrogels from oligopeptides may be chemically cross-linked for increased stability and mechanical strength.^{31,32}

5.3 Hydrogels from Oligopeptides

In a typical strategy for preparing hydrogels from synthetic oligopeptides, precursors self-assemble noncovalently into fibrous nanostructures that form gels through physical cross-linking and entanglements. The key to gel formation is the tuning of molecular and supramolecular interactions to produce a self-supporting network that does not precipitate and is capable of immobilizing water. The noncovalent interactions between self-assembling oligopeptides often mimic the secondary structures responsible for local protein conformation. However, because these interactions generally occur intermolecularly, they also represent a form of quaternary structure.

Most examples of oligopeptide hydrogels utilize the β -sheet motif to produce supramolecular fibers, with only a few recent examples describing the gelation of α -helical peptides and collagen mimetic polyproline helices (CMPs). This is slightly counterintuitive, given that certain natural analogs to α -helical coiled coils and CMPs, keratin and collagen respectively, are components of the hydrogel-like cytoskeleton and extracellular matrix. In contrast, the closest natural analogs of β -sheet oligopeptides are amyloids, protein aggregates with prominent roles in diseases such as Alzheimer's. Another example of hydrogel formation from oligopeptide building blocks is the supramolecular assembly and gelation of peptide amphiphiles (PAs).³³ Although these materials also exhibit some secondary structure, their self-assembly and nanostructures are sufficiently different to warrant separate classification.

5.3.1 β -Sheet Peptide Hydrogels

Zhang *et al.* first observed the gelation of a self-complementary oligopeptide while investigating the EAK16 sequence, Ac-(AEAEAKAK)₂-NH₂, derived from the yeast Z-DNA binding protein *zuotin*.³⁴ The pattern of alternating alanine (A) and glutamic acid (E) or lysine (K) residues promotes the formation of β -strands in which the hydrophobic and charged side-chains are segregated to opposite sides of the peptide backbone. Hydrogen bonding between peptides leads to the formation of intermolecular β -sheets containing a hydrophobic Ala face and a charged Glu/Lys face. Upon addition of salt, the β -sheets further assemble into filaments with diameters of 10–20 nm as a result of hydrophobic bonding between Ala faces and ionic interactions between Glu/Lys faces. The filaments entangle to produce physical hydrogels.

The self-assembly and gelation of EAK16 prompted the development of the rationally designed oligopeptide RAD16 or Ac-(RARADADA)₂-NH₂. The replacement of Glu with aspartic acid (D) and Lys with arginine (R) preserves both the alternating pattern of hydrophobic and charged amino acids and the self-complementarity of the acidic and basic residues.³⁵ As a result, RAD16 also forms intermolecular β -sheets that assemble into filaments and hydrogels. The EAK16 and RAD16 peptides support the attachment of a variety of cell types *in vitro* and form the basis for the commercially available PuraMatrixTM hydrogel system for cell culture and encapsulation.³⁶

Several additional peptide hydrogels are also based on sequences that form β -sheets. For example, Aggeli *et al.* have investigated the secondary structure and self-assembly of two peptides derived from lysozyme and the trans-membrane protein IsK as a function of the solvent dielectric constant and hydrogen bonding ability.³⁷ The resulting phase diagram led to the *de novo* design of DN1 (Ac-QQRFQWQFEQQ-NH₂), an 11-residue peptide that forms antiparallel, intermolecular β -sheets referred to as β -tapes (Q = glutamine, F = phenylalanine, W = tryptophan). Hydrophobic and π - π interactions between aromatic side chains may cause two β -tapes to associate along their hydrophobic faces to form a β -ribbon. TEM reveals helical and twisting behavior in β -tapes and ribbons as well as higher-order self-assembly into fibrils and fibers.^{38,39}

An important consideration in many hydrogel applications is the ability to control the self-assembly process.⁴⁰ For example, the EAK16 and RAD16 family of peptides requires physiological salt concentrations to form hydrogels. In water, electrostatic repulsions between like charges on opposing peptides result in a significant energy barrier for self-assembly. Ions lower this barrier and promote peptide gelation by forming an electric double layer that screens the repulsive charges and permits β -sheet assembly.^{41,42} Using the self-complementary oligopeptide FEK16 or (FEFEFKFK)₂, Messersmith and co-workers have demonstrated controlled assembly of hydrogels by photochemical and thermal release of CaCl₂ from light- and temperature-sensitive liposomes.⁴³ Yu and co-workers have described an alternative approach for controlling self-assembly that involves two complementary peptide sequences, Ac-WKVVKVKVKV-NH₂ and Ac-EWEVEVEVEV-NH₂ (V = valine). Under appropriate conditions, the individual peptides remain in solution because of ionic self-repulsion but assemble into nanofibers and hydrogels upon mixing due to the complementary acidic and basic residues.⁴⁴

The Pochan and Schneider groups have developed an elegant approach to controlling peptide gelation in which the intramolecular folding of a random coil into a β -hairpin is a prerequisite for self-assembly and hydrogel formation (Figure 5.2). Their MAX peptides are derivatives of the rationally designed sequence VKVKVKVKV^DPPTKVVKVKVKV-NH₂.⁴⁵ Whereas the alternating pattern of hydrophobic valine residues and charged lysine residues is a common feature of peptide hydrogels due to its tendency to form β -sheets, the V^DPPT (valine-D-proline-proline-threonine) sequence in the center of this peptide is unique in that it adopts a type II' turn structure. Deprotonation of the charged lysine side chains at pH 9 eliminates electrostatic repulsions and triggers intramolecular folding to produce a β -hairpin with a hydrophobic valine face and a hydrophilic lysine face. Lateral assembly into an extended β -sheet and facial assembly to bury valine residues produces fibrils that further associate into physical hydrogels that are shear-thinning and reversible. Modifications to the primary sequence that alter its hydrophobicity or charge state result in different folding and self-assembly in response to changes in temperature,⁴⁶ ionic strength,⁴⁷ and pH.⁴⁸ Light may also serve as a trigger for β -hairpin formation in a MAX peptide incorporating a photocaged cysteine

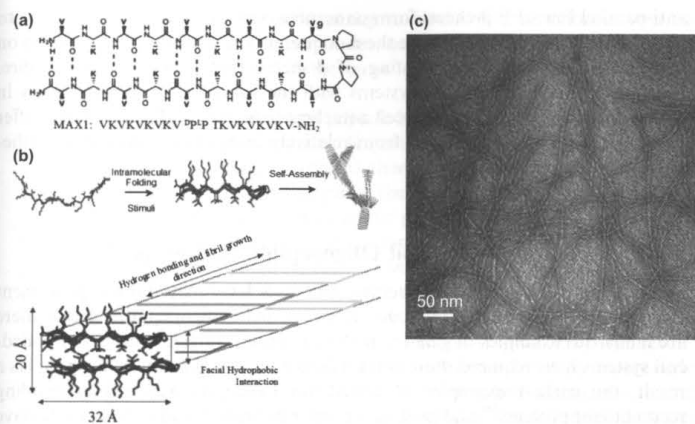


Figure 5.2 Hydrogel formation from the MAX β -hairpin oligopeptide. (a) The MAX peptide contains alternating Val and Lys residues with the potential to form β -hairpins. (b) A stimulus triggers folding of a random coil to a β -hairpin, which can then further assemble into fibrils to form a hydrogel. The proposed mechanism for fibril self-assembly involves burial of the hydrophobic Val face and lateral association of hairpins through intermolecular hydrogen bonding. (c) Fibrils can be observed in a diluted hydrogel by transmission electron microscopy. (Reproduced from Ozbas *et al.*⁴⁷ by permission of the American Chemical Society.)

residue.⁴⁹ While the replacement of a valine residue with an α -carboxy-2-nitrobenzyl-protected cysteine (CNB-Cys) prevents intramolecular folding, cleavage of the protecting group by UV irradiation results in peptide self-assembly and gelation. Thus, in addition to demonstrating a novel method for controlling hydrogel formation, this work also highlights the utility of incorporating two non-natural amino acids, D-Pro and CNB-Cys, into hydrogel precursors.

The examples considered thus far involve the gelation of oligopeptides with lengths of 10–20 amino acids. An interesting question is how small such precursor peptides can be while still retaining the ability to self-assemble into hydrogels. A number of research groups are addressing this question in their investigations of low molecular weight hydrogelators (LMWG).⁵⁰ In these systems, fibrous hydrogels assemble from short peptide sequences, typically dipeptides and tripeptides, conjugated to large aromatic groups such as fluorenylmethoxycarbonyl (Fmoc). Triggers for LMWG self-assembly include acidification to protonate the C-terminus,^{51,52} temperature change,⁵³ solvent change,⁵⁴ and enzymatic activity.^{55,56} The best studied example of a LMWG is the FmocPhePhe dipeptide. Spectroscopic measurements indicating β -sheet formation and π - π stacking have led to a model for self-assembly in which

anti-parallel FmocFF β -sheets form nanotubes 3 nm in diameter.⁵⁷ The keys to nanotube formation appear to be the π - π interactions between Fmoc groups on adjacent β -sheets and the twisting of β -sheets that leads to tube closure. Functionalization of LMWG systems with the FmocRGD peptide results in hydrogel matrices that support cell attachment *in vitro*.⁵⁸ These systems offer the potential to form hydrogels from relatively inexpensive and easily synthesized starting materials.

5.3.2 α -Helical Coiled-Coil Oligopeptide Hydrogels

When compared to β -sheets, α -helical structures have played a less prominent role in the development of hydrogels from synthetic oligopeptides. While there are numerous examples of gelating β -sheet peptides, similar α -helical or coiled-coil systems have required more careful design to assemble into hydrogels. As a result, the earliest examples of coiled-coil hydrogels were self-assembling recombinant proteins⁵⁹ and protein-polymer hybrids,⁶⁰ and only recently have research groups reported the gelation of synthetic oligopeptide coiled coils.^{61–63}

Coiled-coil peptides are not true α -helices but are closely related.⁶⁴ The name derives from the fact that two helices or coils wrap around one another to form a dimer, although higher-order oligomers are also possible. Rather than the expected 3.6 residues per turn for an α -helix, coiled coils complete 3.5 residues per turn. A common description of coiled-coil peptide sequences uses an *abc-defg* heptad repeat notation, with seven residues representing two turns of the helix (Figure 5.3a). The residues at positions *a* and *d* are nonpolar and form a hydrophobic face to facilitate contact with the other coil (or coils in the case of multimers). Positions *e* and *f* are frequently charged residues while *b*, *c*, and *g* are polar. Coiled coils are important motifs in natural proteins, including

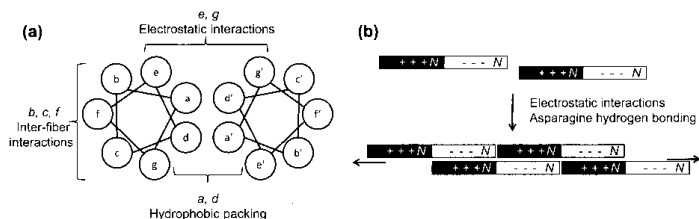


Figure 5.3 Coiled-coil peptides and fibers. (a) Helical wheel representation of a coiled-coil dimer. Dimer formation is driven by hydrophobic association of the *a* and *d* residues and is often stabilized by electrostatic attractions at the *e* and *f* positions. The *b*, *c*, and *f* positions have important roles in controlling interactions between dimers that govern fiber thickening and hydrogel formation. (b) Staggered dimerization leading to fiber formation can be enforced by electrostatic interactions (*e* and *f*) and asparagine hydrogen bonding on the hydrophobic face.

transcription factors that must dimerize to bind DNA, and fibrous structural proteins such as keratin. The preponderance of leucine at the *d* position of the heptad repeat has given rise to the term “leucine zipper” to describe certain coiled coils.⁶⁵

The first step toward coiled-coil peptide hydrogels involves the self-assembly of oligopeptides into longer fibers. Kajava and co-workers have formalized rules for fiber formation and applied them to the *de novo* design of homopentamer coils.⁶⁶ Their design employs the staggered assembly of coils in the axial direction to produce fibers at acidic pH. Zimenkov *et al.* have adopted some of these rules to form fibers from dimeric coiled coils using a precursor peptide containing six heptad repeats.⁶⁷ Electrostatic interactions at the *e* and *f* positions and a hydrogen bond between asparagine residues on the hydrophobic face enforce parallel dimerization between three N-terminal heptads of one strand and three C-terminal heptads of another (Figure 5.3b). The result of this staggered arrangement is a dimer with “sticky ends” that promotes coiled-coil extension into nanofibers that thicken through lateral association. Using a similar strategy, the Woolfson group has developed heterodimeric coiled-coil peptides that self-assemble into fibers upon mixing.^{68,69} Finally, Hartgerink and co-workers have demonstrated the formation of uniform fibers from blunt-ended homodimers with increasing peptide concentrations and acidification.⁶² A recent review provides additional examples of coiled-coil fiber formation.⁷⁰

Although there are now several examples of fibers produced by extending coiled-coil peptides, only a few have led to hydrogel formation. The Woolfson group has engineered their self-assembling fiber system to form hydrogels by controlling fiber thickening.⁶¹ They accomplish this by careful choice of amino acids at the *b*, *c*, and *f* positions of the coiled-coil heptad. These residues have a minimal effect on coil dimerization but mediate the lateral association between multiple dimers in solution. Whereas strong electrostatic interactions result in thick fibers that settle out of solution,⁷¹ weaker interactions can create cross-link points for a physical hydrogel. When all three residues are glutamine, weak gels form but melt as the temperature increases due to a loss of hydrogen bonding. Conversely, peptides with alanine at all three positions form gels that strengthen with increasing temperature, an observation that is consistent with physical cross-linking mediated by hydrophobic interactions.

The blunt-ended coiled-coil dimers of the Hartgerink group also form reversible, self-supporting gels when sufficiently concentrated.⁶² The proposed mechanism of gel assembly involves protonation of glutamic acids in the *e* position to reduce electrostatic repulsions. Dexter and co-workers have described the similar pH-dependent gelation of a coiled-coil peptide that features an expanded hydrophobic face at the *a*, *d*, *e*, and *g* positions and likely assembles as a homohexamers.⁶³ Charged residues at the *b*, *c*, and *f* positions appear to affect the assembly of coiled-coil fibers into hydrogels, with an overall charge of +1 or -1 required for gelation. Thus, while examples of hydrogels from coiled-coil oligopeptides remain rare compared to β -sheet hydrogels, these recent reports indicate that design rules for this system are beginning to emerge.

5.3.3 Collagen Mimetic Peptide Hydrogels

An emerging class of peptide hydrogels is based on the triple-helical structure of collagen and its assembly into higher-order fibrous structures. With at least 28 isoforms, collagens are the most abundant proteins in vertebrates and are responsible for much of the mechanical integrity of the extracellular matrix.⁷² While naturally derived sources of collagens are common materials for cell culture and tissue engineering, a synthetic substitute would be more homogeneous and eliminate the potential for contamination from animal tissues. To realize this goal, several research groups have explored the molecular and supramolecular structures of collagen mimetic peptides, or CMPs.^{73,74}

Early efforts in this field sought to analyze the assembly and stability of triple-helical CMPs as a model system for collagen. The most common CMP sequences are repeats of the Gly-Pro-Pro (GPP) or Gly-Pro-Hyp (GPO) tripeptides, where Hyp (O) is hydroxyproline. These primary sequences adopt a left-handed polyproline type II helical structure, and three such helices associate as a right-handed superhelix. The requirement for Gly in CMPs stems from the steric hindrance encountered by larger side chains in the center of the helix, while Pro residues promote the preorganization of helical strands and Hyp contributes to stability through solvation or stereoelectronic effects.⁷² While researchers have gained important structural information from these first-generation CMPs, evidence for their higher-order assembly into fibers and gels has been limited.⁷⁵

As with coiled-coil peptides, progress toward higher-order CMP structures requires the extension of triple helices in the axial direction followed by their lateral and head-to-tail association into fibers. Kotch and Raines have accomplished axial extension using disulfide bonds to create cysteine-knotted trimers with single- and double-stranded overhangs at the termini.⁷⁶ The overhangs associate with the appropriate strands on opposing molecules to extend the triple helix into nanofibers. The Koide group has also developed a cysteine knot method for producing long triple-helical CMPs that form gels.^{77,78} Chaikof, Conticello and co-workers have described the formation of the most collagen-like synthetic fibers to date using a zwitterionic CMP, (PRG)₄(POG)₄(EOG)₄.⁷⁹ Electrostatic interactions between Arg and Glu side chains produce triple helices that further assemble upon thermal annealing into 70 nm wide fibers with periodic banding patterns similar to those in native collagen. Alternative strategies to produce long triple helices involve chain extension of telechelic CMPs functionalized with thiols and thioesters for native chemical ligation,⁸⁰ ligands for metal-ion coordination,^{81,82} and aromatic rings for π - π stacking and cation- π interactions.^{83,84}

The Hartgerink group has reported the most comprehensive study to date on the hierarchical assembly of CMPs into hydrogels (Figure 5.4).⁸⁵ Adopting an electrostatic sticky-end approach similar to that of Chaikof and Conticello, they have demonstrated triple helix formation by a zwitterionic CMP, (PKG)₄(POG)₄(DOG)₄. They hypothesize that replacement of Arg with Lys and of Glu with Asp results in more effective salt bridges and improved higher-order

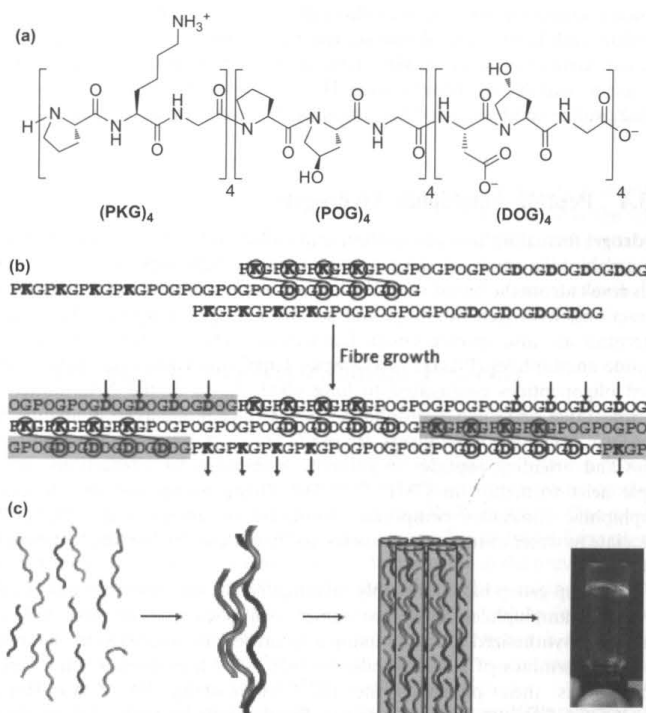


Figure 5.4 Hierarchical assembly of collagen-mimetic peptides. (a) The CMP from O'Leary *et al.*⁸⁵ contains Lys and Asp residues that stabilize triple helix formation through salt bridges. (b) "Sticky-ended" CMP triple helices form fibers in the axial direction. Charged residues that do not participate in intrahelical salt bridges may promote lateral association of helices. (c) The consequence of this design is the formation of long triple-helical CMP fibers that further assemble into lateral bundles and entangled hydrogel networks. (Reproduced from O'Leary *et al.*⁸⁵ by permission of Macmillan Publishers.)

assembly. Nanofibers of width 4–5 nm are consistent with bundling of multiple triple helices that further assemble into collagenase-degradable hydrogels.

A future challenge in the field of CMP hydrogels is the development of properly assembling precursor peptides that lack hydroxyproline. While Hyp is important for triple helix stability, it has limited researchers to assembling long polypeptide chains from short, synthetic oligopeptides rather than recombinant

proteins. Using a combination of electrostatic stabilization and cysteine knots, Krishna and Kiick have demonstrated the formation of triple helices and fibrillar structures from CMPs lacking Hyp.⁸⁶ Engineering recombinant organisms capable of incorporating Hyp during protein synthesis⁸⁷ or modifying proline residues post-translationally⁸⁸ would offer alternative solutions.

5.3.4 Peptide Amphiphile Hydrogels

Hydrogel formation in many β -sheet and coiled-coil systems is due in part to the amphiphilic nature of the precursor peptides. Self-assembly in such materials results from the burial of apolar regions and a combination of electrostatic attractions, hydrogen bonding, and solvation in polar regions. These characteristics are also specific design features of a class of materials known as peptide amphiphiles (PAs). For example, Tirrell and co-workers have synthesized oligopeptides conjugated to long alkyl chains at the N-terminus and demonstrated that these hydrophobic tails produce monolayers at an air/water interface.⁸⁹ The PA monolayers are useful for displaying cell adhesion molecules and orienting peptides to promote intermolecular interactions such as triple helix formation in CMPs.^{90,91} The Zhang group has also developed amphiphilic molecules composed completely of amino acids. These PAs associate in water to form nanovesicles and nanotubes by burying hydrophobic residues.⁹²

The Stupp group has thoroughly investigated the self-assembly and gelation of peptide amphiphiles, as well as numerous applications for these materials. They have synthesized PAs containing a 16-carbon alkyl tail covalently bonded to the N-terminus of a hydrophilic peptide.^{93,94} An example of this type of molecule is their phosphoserine ($S^{(PO_4)}$)-containing PA, $CH_3(CH_2)_{15}O-CCCCGGGS^{(PO_4)}RGD$ (C =cysteine). Since the hydrophobic tail is slightly narrower than the peptide head, PAs tend to form cylindrical micelles in water. Upon slow acidification or addition of calcium ions, PAs self-assemble into nanofibers that gel at sufficiently high concentrations. Oxidation of cysteine residues creates reversible disulfide cross-links that stabilize PA nanostructures. Alternatively, UV-mediated cross-linking can occur between hydrophobic tails that contain diacetylene moieties rather than simple alkyl chains.³² Applications of PA hydrogels include their use as tissue engineering scaffolds and drug delivery vehicles.⁹⁵ The Stupp group's original peptide amphiphiles incorporated phosphoserine residues that act as templates for the mineralization of hydroxyapatite, as observed in bone formation.⁹³ Nanofiber and hydrogel formation have proven robust to significant sequence variation in the peptide region, permitting incorporation of a variety of biologically active peptide domains, including those with considerable hydrophobic content. Examples include PAs displaying the cell-binding peptides RGD and IKVAV, growth factor-mimicking epitopes, and heparin-binding peptides (I = isoleucine).^{94,96,97}

Deming and co-workers have produced rapidly recovering hydrogels from a different class of peptide amphiphiles.^{98,99} Using *N*-carboxyanhydride (NCA)

polymerization, they prepared long diblock copolypeptides of the form $K_{160}L_{40}$ and $K_{160}V_{40}$ with very low polydispersity. The hydrophobic blocks adopt α -helical and β -sheet structures when constituted from leucine (L) and valine, respectively. In the case of poly-leucine helical hydrophobic blocks, the proposed model for self-assembly involves perpendicular alignment of α -helices in a twisted fibril that is surrounded by a polyelectrolyte "brush".¹⁰⁰ This is in contrast to other self-assembling helical peptides in which alignment occurs parallel to the helical axis. Gelation occurs above a concentration threshold that is dependent on the length of the hydrophilic block as well as the length and structure of the hydrophobic block. Triblock ($K_3L_3K_3$) and pentablock ($K_3L_3K_3L_3K_3$) copolypeptides also self-assemble and form gels, with pentablocks having the potential for intermolecular cross-links between fibrils.¹⁰¹

5.4 Hydrogels from Recombinant Proteins

Recombinant proteins are another class of building blocks for engineered hydrogels. Whereas oligopeptides prepared by SPPS are limited in length and polypeptides prepared NCA polymerization are limited in sequence diversity, recombinant expression exploits the ability of a host organism to synthesize full-length proteins of almost any sequence from DNA templates. One result of this is a fundamental difference in the mechanisms for hydrogel formation by oligopeptides *versus* recombinant proteins. As discussed in the previous section, short oligopeptides self-assemble into nanofibers that entangle to form hydrogels. Proteins, on the other hand, can be long enough to form hydrogel networks without preassembling into fibers. Instead, network formation is driven by noncovalent interactions between physical cross-linking domains or by covalent bonds between chemically cross-linked residues.

Protein engineering offers the potential for unprecedented control over hydrogel structure and functionality.^{2,3} Two important categories of recombinant proteins for hydrogels are self-assembling artificial proteins and biomimetic proteins based on elastins and silks. Recent advances in protein engineering have also led to the development of a third category that combines assembly domains with full length, functional proteins to form multifunctional hydrogels.¹⁰²

5.4.1 Self-Assembling, Multidomain Artificial Proteins

Our laboratory has investigated recombinant artificial proteins as precursors for self-assembling hydrogels based on the association of coiled-coil domains.⁵⁹ Telechelic triblock proteins denoted AC_xA contain two leucine-zipper domains (A) flanking a random coil polyelectrolyte chain, $(AG)_3PEG$, repeated x times (C_x). Variation in the association of zipper domains in response to temperature and pH changes results in a reversible sol-gel transition. At high temperature or high pH, solutions of AC_xA behave as viscous liquids. Decreasing the temperature to refold the helices, or lowering the pH to near-neutral values, leads

to strong association of leucine-zipper domains as tetramers. If the polyelectrolytic linker region remains solvated, coiled-coil tetramers can act as physical cross-links in a hydrogel network. Using genetically engineered endblocks, Kopeček and co-workers have demonstrated that the oligomerization state, self-assembly behavior, and pH and temperature responsiveness of coiled-coil hydrogels can be tuned by altering the amino acid sequence.^{103,104}

Hydrogels assembled from AC_xA triblocks are unexpectedly soft and erode rapidly in open solutions, thus limiting their potential applications.^{105,106} This behavior is due to the tendency of AC_xA to form non-productive loops that do not contribute to the elasticity of the network. The network is transient and rapid exchange of peptide strands between coiled-coil aggregates allows zippers to disengage and dissolve in the surrounding medium. Our group has demonstrated three strategies that overcome these limitations and produce stiffer hydrogels with tunable erosion rates. The first is to stabilize leucine-zipper aggregates through disulfide bond formation (Figure 5.5a).¹⁰⁶ By the judicious placement of cysteine residues in the zipper domains, it is possible to preferentially stabilize intermolecular aggregates while allowing exchange of looped strands. In an alternative strategy, longer polyelectrolyte linkers suppress loops that arise to avoid energy penalties associated with stretching shorter chains (Figure 5.5b).¹⁰⁵ Unlike typical elastic materials that become softer upon increasing the molecular weight between cross-links, AC_xA hydrogels can become stiffer when the linker region is extended. Finally, redesigned triblock proteins of the form AC_xP and PC_xP (Figure 5.5c), where P is a zipper domain derived from the cartilage oligomeric matrix protein, are stiffer and exhibit slower erosion rates than AC_xA .¹⁰⁷ In the case of PC_xP , these observations arise from the higher aggregation number and parallel alignment of the P zippers. While A zippers can adopt an antiparallel orientation as part of tetrameric coiled coils, P zippers associate exclusively in a parallel orientation in pentameric aggregates. This results in a lower tendency for PC_xP to form intramolecular loops, as the linker region must stretch to permit the proper parallel alignment. In the case of AC_xP triblocks, intramolecular loops rarely form due to the preference of the A and P zippers to aggregate as homo-oligomers rather than hetero-oligomers.

The ability to tune the assembly and erosion of telechelic leucine-zipper hydrogels suggests a potential application as materials for the encapsulation and controlled release of cells or biomolecules. In this respect, desirable features include shear-thinning behavior and the rapid recovery of elastic strength upon cessation of shear. These properties would permit the delivery of the hydrogel and its cargo *via* a minimally invasive injection. Rheological measurements of PC_xP hydrogels indicate a decrease in the elastic modulus of three orders of magnitude at high strain rates (Figure 5.6a).¹⁰⁸ Hydrogels also recover their elastic strength within seconds after large-amplitude oscillatory strain and form self-supporting structures when injected through narrow gauge needles (Figure 5.6a,b). Shear-thinning appears to be due to yielding behavior within the gel, with shear banding potentially protecting cells and biomolecules from high shear rates. The nonlinear rheology of PC_xP hydrogels is largely unaffected by

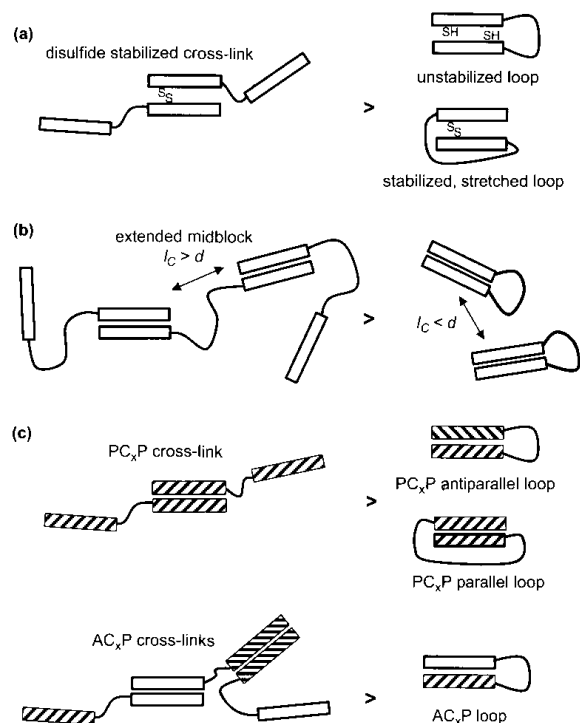


Figure 5.5 Stabilization of self-assembling artificial proteins to control hydrogel stiffness and erosion rate. Cross-links are depicted as dimers for clarity, although tetrameric and pentameric coiled-coils predominate in these systems. (a) Disulfide formation between cysteine residues in the AC_xA domain stabilizes intermolecular cross-links. The protein design precludes stabilization of antiparallel intramolecular loops, while parallel loops rarely form due to chain stretching. (b) Cross-linking is favored by an extended midblock C with a mean end-to-end distance (l_c) greater than the average distance between proteins (d). In contrast, shorter midblocks form loops to avoid energy penalties associated with chain stretching. (c) Proteins of the form PC_xP contain P coil domains that aggregate exclusively in a parallel orientation. Cross-linking is preferred to loop formation, which requires chain stretching. Proteins of the form AC_xP do not form intramolecular loops because the A and P domains (white rectangles and striped rectangles, respectively) do not form hetero-oligomers.

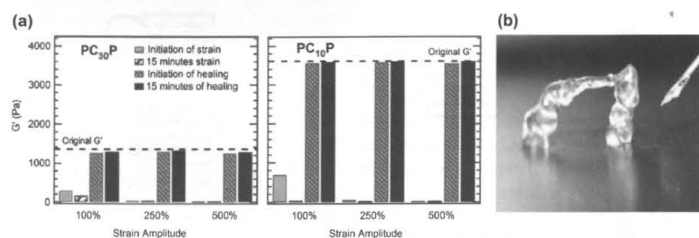


Figure 5.6 Shear-thinning and elastic recovery of PC_xP hydrogels. (a) Hydrogels experience a decrease in their shear storage modulus (G') of three orders of magnitude at the onset of large-amplitude oscillatory strain but recover their elastic strength within seconds. Recovery was independent of the length of the midblock in PC₁₀P and PC₃₀P hydrogels. (b) PC_xP forms a self-supporting gel upon injection through a 22 gauge needle. (Reproduced from Olsen *et al.*¹⁰⁸ by permission of the American Chemical Society.)

the length of the polyelectrolyte linker region, suggesting that it should be possible to incorporate biologically active sequences into shear-thinning telechelic proteins.¹⁰⁹

Telechelic proteins containing collagen-mimetic endblock sequences also produce shear-thinning, thermoreversible hydrogels.^{110,111} The triblock architecture of these proteins consists of nine repeats of a Pro-Gly-Pro sequence flanking a central random coil. Recombinant production in yeast yields gram-per-liter quantities of the secreted product.¹¹² In these materials, endblock aggregation occurs through formation of triple helices. Hydrogels assembled from CMP telechelic triblocks have shown promise in the controlled release of model proteins.¹¹³ Chemical cross-linking of lysine residues in the random coil midblock using glutaraldehyde produces shape-memory hydrogel networks that are dependent on the thermoreversibility of the endblock triple helices.¹¹⁴

Many peptide hydrogels require temporary exposure to non-physiological pH, temperature, or ionic strength in order to self-assemble. These conditions may result in unacceptable levels of cell death or denaturation of encapsulated cargo. To overcome these problems, Heilshorn and co-workers have introduced the mixing-induced, two-component hydrogel (MITCH) system in which gelation can occur only upon mixing of two protein solutions.¹¹⁵ The two components of MITCH gels are artificial proteins consisting of several repeats of either the WW or proline-rich domains separated by random coil linker regions. Physical cross-linking between proteins containing the WW domain, named for its conserved tryptophan residues, and proteins containing the proline-rich domain derives from the noncovalent association between these sequences as found in natural proteins. By varying the stoichiometry of the two components as well as the frequency and binding strength of the cross-linking domains, it is possible to tune the sol-gel transition and viscoelastic properties

of the system in a predictable manner.^{115,116} The transient nature of the network results in shear-thinning protein hydrogels that display rapid self-healing within minutes after injection.

5.4.2 Biomimetic Recombinant Proteins: Elastins and Silks

Elastins are important components of the extracellular matrix and represent attractive targets for biomedical engineering applications. The desirable mechanical and chemical properties of elastins can be recapitulated in elastin-like polypeptides (ELPs) containing repeats of the VPGXG pentamer, where the "guest residue" X can be any amino acid except proline. Elastin-like polypeptides exhibit inverse temperature behavior in that they are soluble at low temperatures but aggregate in coacervate phases at higher temperatures. The transition from a soluble to an aggregated state occurs at the lower critical solution temperature (LCST), where it has been proposed that ELPs transition from a random coil conformation to a β -spiral. The LCST is dependent on the length of the sequence, the ELP concentration, and the hydrophobicity of the guest residue, with more hydrophilic sequences exhibiting higher LCSTs.^{117,118} While highly repetitive ELPs can be produced synthetically, recombinant engineering permits access to more complex protein/sequences, including multiblock architectures and bioactive domains.^{119,120} One of the most common engineering strategies is to vary the ELP guest residue to incorporate reactive moieties for chemical cross-linking or to alter the LCST behavior of the protein. Temperature cycling above and below the LCST offers a facile method of purification of recombinant ELPs.¹²¹

Elastin hydrogels have been formed by both physical and chemical cross-linking methods. Conticello and co-workers have described the physical cross-linking of triblock ELPs based on inverse temperature transitions.^{122,123} The mechanism for gel formation is similar to that of telechelic leucine-zipper and CMP hydrogels. Triblocks of the form BAB contain hydrophobic endblock elastin sequences (B) flanking a hydrophilic elastin midblock (A). At temperatures above their LCST, endblocks undergo microphase separation while the hydrophilic midblock (which has a higher LCST) remains solvated. This results in hydrogels in which the aggregated hydrophobic blocks serve as thermoreversible, noncovalent cross-linkers. Chilkoti and co-workers have analyzed the rheological behavior of an ELP above its LCST. Although their design did not include explicit cross-linking domains, they observed gel-like behavior in the coacervate with potential applications in cartilage tissue engineering.¹²⁴

Several research groups have employed chemical cross-linking to form hydrogels with mechanical properties that more closely resemble those of native elastin. The most common strategy uses small multifunctional cross-linkers that form covalent bonds with reactive amino acids, typically lysines occupying the ELP guest site or in distinct cross-linking domains.¹²⁵⁻¹³¹ Other strategies include enzymatic cross-linking,¹³² UV or visible light photo-cross-linking,^{27,133} and γ -irradiation.¹³⁴

Hydrogels from silk proteins represent another class of potentially useful biomimetic materials.¹³⁵ The best studied silk proteins are those from the Chinese silkworm *Bombyx mori* and from the dragline of the spider *Nephila clavipes*. Fibers spun from silk proteins derive their impressive mechanical strength and extensibility from repetitive Gly- and Ala-rich β -sheet crystalline domains separated by amorphous hydrophilic linker regions. Kaplan and co-workers have demonstrated that solutions of naturally derived silkworm fibroin doped with poly(ethylene glycol) undergo a sol-gel transition over the course of days due to the coalescence of hydrophobic regions.^{136–138} Hydrogels produced from silk fibroin are candidates for tissue engineering and cell encapsulation applications.^{139,140}

In contrast to extensively engineered ELPs, silk protein engineering has generally focused on producing close facsimiles of the natural silks. In particular, there has been strong interest in developing recombinant sources of dragline spider silk since spiders, unlike silkworms, are not easily farmed. Several research groups have demonstrated progress in this area by producing spider silks in bacterial,¹⁴¹ plant,¹⁴² mammalian,¹⁴³ and silkworm hosts.¹⁴⁴ While significant attention has been directed toward spinning recombinant silks into fibers, only a few studies have examined hydrogel formation from these proteins.^{145,146} Further protein engineering could offer the ability to tune the mechanical properties of silk and silk hydrogels or to introduce new bioactive domains.^{147,148} An alternative approach to silk-like hydrogels involves genetically engineered block copolymers of silk-like GAGAGS (S = serine) peptides and elastin-like VPGXG peptides.^{149,150} Solutions of these proteins spontaneously form swollen hydrogels that display the crystalline hydrophobic domains of silks and the elasticity and responsiveness of ELPs. These materials have shown promise as delivery vehicles for gene therapy.¹⁵¹

5.4.3 Multifunctional Protein Hydrogels

Many proposed applications of peptide hydrogels in tissue engineering and drug delivery require materials that display simple binding motifs and assemble or disassemble in response to environmental cues. Both oligopeptides and recombinant proteins have shown promise in meeting these requirements. However, protein engineering also offers the ability to develop hydrogels with much more advanced functionality. In this regard, emerging research has demonstrated the potential of engineering multifunctional protein hydrogels and their applications as sensors and catalytic materials.

Banta and co-workers have developed multifunctional protein hydrogels that combine the self-assembly behavior of leucine zippers with the useful functional properties of fluorescent proteins and enzymes. In their initial demonstration of this approach, they showed that the incorporation of green fluorescent protein in the midblock region of AC₄A does not inhibit protein folding or gel formation.¹⁵² Using this method, they produced multicolor hydrogels and probed gel structure in these systems by Förster resonance energy transfer (FRET). A similar design that incorporated an oxidase enzyme and electron conductors

was used to create bioelectrocatalytic hydrogels capable of reducing molecular oxygen to water.¹⁵³ The electron-conducting hydrogel network consisted of triblock leucine-zipper proteins with osmium bis-bipyridine complexes bound to histidine residues. Enzymatic activity was derived from a chimeric protein containing a leucine-zipper domain and the small laccase (SLAC) polyphenol oxidase from *Streptomyces coelicolor*. The zipper domain mediates incorporation of the chimera into the hydrogel network, while dimerization of the SLAC enzyme provides additional cross-linking and is required for catalytic activity. This novel class of materials has potential applications in biofuel cells and oxygen sensors. Other examples of enzymatic hydrogels incorporate an aldo-keto reductase and an organophosphate hydrolase, suggesting the broad applicability of this method.^{154,155}

In similar work, Gallivan and co-workers have engineered a chimeric calmodulin protein fused to a leucine-zipper domain.¹⁵⁶ Calmodulin is an important regulatory protein that undergoes a conformational change in the presence of calcium ions to bind partner proteins. A solution of the chimeric calmodulin-zipper protein and a telechelic cross-linker containing calmodulin-binding endblocks forms a hydrogel network in the presence of Ca²⁺. The network is reversible upon chelation of calcium.

5.5 Hydrogels from Peptide-Polymer and Protein-Polymer Hybrids

As discussed earlier in this chapter, a common design for recombinant protein hydrogels is a telechelic triblock architecture with physical cross-linking domains separated by a polyelectrolyte linker. Hydrogel formation results from an appropriate balance between endblock aggregation and linker solubility. While genetically encoded linkers offer clear advantages such as monodispersity, sequence diversity, and bioactivity, synthetic polymers can also fill this role in hydrogels formed from peptide-polymer and protein-polymer hybrids. From a synthesis and cost perspective, hydrogels in which the bulk of the dry weight derives from synthetic macromolecules may be more desirable than hydrogels from oligopeptides or recombinant proteins. Furthermore, whereas ribosomal translation limits recombinant proteins to linear architectures, synthetic methods offer access to branched architectures such as graft, star, and dendritic polymers.

In hybrid hydrogels, peptides and proteins may serve several roles. Most commonly, they are responsible for chemical or physical cross-linking of the prepolymer solution. The mechanisms for cross-linking and self-assembly are analogous to those described for recombinant protein hydrogels. Peptides and proteins may also confer biological activities such as cell binding and enzymatic degradability to otherwise inert polymers. Finally, proteins embedded in hybrid hydrogel networks may exhibit enzymatic activity or ligand binding for applications in stimulus-responsive materials and biosensors. We present several examples of hybrid hydrogels as an introduction to this field. A more

detailed account of protein- and peptide-polymer materials is provided by Krishna and Kiick.¹⁵⁷

5.5.1 Peptides as Physical and Chemical Cross-Linkers

Kopeček and co-workers have pioneered the development of physically cross-linked hybrid hydrogels from proteins and synthetic polymers.⁶⁰ Their design features recombinant coiled-coil proteins attached to a copolymer of *N*-(2-hydroxypropyl)methacrylamide and (*N*',*N*'-dicarboxymethylaminopropyl)methacrylamide. The attachment is mediated by Ni²⁺ coordination by the polyhistidine tag on the protein and the iminodiacetate side chains of the copolymer. As in triblock leucine-zipper recombinant proteins, coiled-coil aggregation leads to hydrogel formation. A second generation design features two copolymers covalently grafted with coiled coils that associate as heterodimers.¹⁵⁸ Reversible hydrogels form upon mixing the two copolymers at neutral pH at concentrations as low as 0.1 wt%.

Shen and co-workers have further demonstrated the cross-linking potential of coiled coils in a bottom-up approach to fabricating hydrogels for tissue engineering.¹⁵⁹ Using photolithography, they encapsulate cells in 400 μ m star-shaped microgels by photopolymerization of poly(ethylene glycol) (PEG) diacrylate. Subsequent attachment of cysteine-containing coiled coils to the residual acrylate groups facilitates reversible assembly of microgels into porous macroscopic scaffolds. The high porosity and pore interconnectivity of these scaffolds and the short length scales of the microgels may aid nutrient transport to the encapsulated cells.

The Yu group has investigated hydrogel formation from four-arm PEG stars functionalized with collagen-mimetic peptides.¹⁶⁰ The association of CMPs as triple helices forms a network that is reversible by heating above the melting temperature and addition of competitor CMPs. They exploit this reversible behavior to form gradients in gel stiffness through local injection of a hot CMP solution. As the solution diffuses from the injection site, the thermal gradient melts the triple helical cross-links, which do not reform upon cooling due to the high concentration of competitor peptides. They have also demonstrated the use of CMPs to modify natural collagens noncovalently with PEG and growth factors through a strand invasion mechanism.^{161,162} These systems highlight the potential of hybrid hydrogels containing synthetic polymers, peptides, and naturally derived proteins.

Covalent cross-linking offers another route to peptide polymer hydrogels (Figure 5.7). Hubbell and co-workers have pioneered the development of peptide- and protein-functionalized PEG hydrogels using photopolymerization of acrylated precursors and Michael-type addition reactions.^{163–165} Both methods have been used to introduce cell-binding ligands and protease-sensitive peptide sequences for enzymatic degradation by plasmin and matrix metalloproteases.^{166,167} Anseth and co-workers have extended this method for cell encapsulation under mild conditions using the bioorthogonal strain-promoted azide-alkyne cycloaddition (SPAAC). To accomplish this, they have

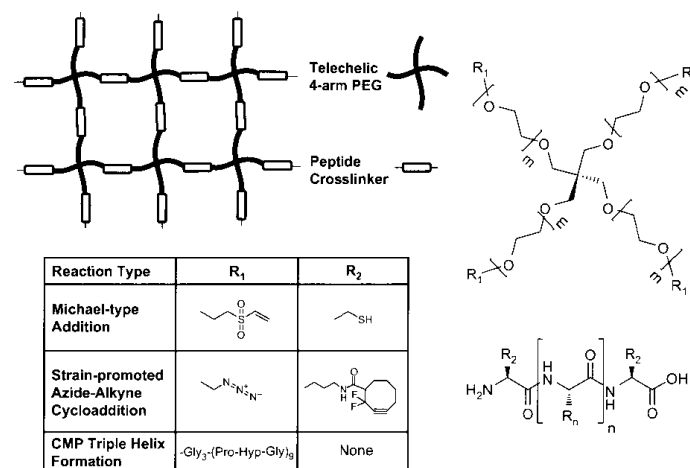


Figure 5.7 Chemical and physical cross-linking of four-arm PEG stars. Telechelic four-arm PEG is functionalized with vinyl sulfone and azide groups for Michael-type addition and strain-promoted azide-alkyne cycloaddition, respectively. Chemical cross-linking occurs through difunctional peptides that contain terminal cysteines or cyclooctynes. Alternatively, physical hydrogels are formed by functionalizing four-arm PEG with collagen-mimetic peptides that associate noncovalently as triple helices.

cross-linked tetraazide-functionalized four-arm PEG stars with peptides containing terminal cyclooctynes.²² Their peptide cross-linker also contains an alkene moiety for subsequent photopatterning of cell-binding ligands by the thiol-ene reaction and a nitrobenzyl ether moiety for controlled photodegradation.¹⁶⁸

5.5.2 Synthetic Hydrogels Containing Functional Protein Domains

In parallel with the development of self-assembling, multifunctional hydrogels from recombinant proteins, several groups have demonstrated the potential of incorporating functional proteins such as enzymes and antibodies into synthetic polymer scaffolds. For example, two groups have produced calcium-responsive materials through the covalent incorporation of calmodulin (CaM) into cross-linked polyacrylamide (PA) and PEG scaffolds.^{169,170} Both networks undergo contraction in the presence of Ca²⁺ and swelling upon its chelation. In the case of Ehrick *et al.*, contraction of the PA hydrogel results from binding between CaM and a similarly immobilized CaM ligand. This material has been

used to create chemically tunable microlenses.¹⁷¹ In the system developed by Murphy *et al.*, conformational changes in CaM drive contraction of the PEG scaffold. This system has been used for the controlled release of encapsulated growth factors.¹⁷²

Other examples of functional protein polymer hybrids include hydrogels that swell and contract due to antigen-antibody binding¹⁷³ and catalytic activity of grafted enzymes.¹⁷⁴ In addition, there are several examples in which growth factors are covalently cross-linked to a polymer scaffold for tissue engineering applications.¹⁷⁵⁻¹⁷⁷ Although the protein components do not necessarily contribute to hydrogel assembly or function, these systems still represent an important class of hybrid materials.

5.6 Future Directions and Challenges

In the past 20 years, the field of peptide and protein hydrogels has grown from early observations of self-assembly of oligopeptides to the design of advanced materials with well-controlled biological activity and mechanical properties. Hydrogels are now routinely produced from oligopeptides, recombinant proteins, and peptide polymer hybrids. Together with hydrogels from naturally sourced biomolecules and synthetic polymers, these materials provide scientists, engineers, and clinicians with a multitude of options to address problems in medicine and basic biology. In this regard, an important challenge will be to match the most appropriate materials with each application, particularly when transitioning hydrogels into clinical settings. Other challenges include assessing the immune response to peptide and protein hydrogels and producing hydrogel precursors in sufficient quantity and purity for their intended applications. These and other challenges will guide the design of future generations of engineered hydrogels.

5.6.1 Immune Response to Peptide and Protein Hydrogels

Peptide and protein hydrogels are frequently touted as biocompatible on the basis of the fact that their precursors resemble natural biopolymers and should be susceptible to enzymatic or hydrolytic degradation. However, implantation of hydrogels still has the potential to generate undesirable immune responses. While more thorough investigations will be required, initial *in vivo* and *in vitro* studies to analyze the immunogenicity of peptide hydrogels and their precursors have been encouraging. For example, using the RAD16 and EAK16 peptides, Holmes *et al.* detected no inflammatory response after intramuscular injection in rats and no measurable antibody response when the peptides were conjugated to bovine serum albumin and injected in rabbits and goats.¹⁷⁸ Similarly, myocardial injection of RAD16 hydrogels in mice did not generate significant inflammation,¹⁷⁹ while the MAX β -hairpin hydrogels of the Schneider and Pochan groups did not elicit an inflammatory response from macrophages in an *in vitro* assay.¹⁸⁰ Promising results have also been reported for elastin-like and silk-elastin-like materials.^{181,182}

The Collier group has further investigated the immunogenicity of both coiled-coil and β -sheet self-assembling oligopeptides.¹⁸³⁻¹⁸⁵ They have demonstrated in a mouse model that β -sheet oligopeptides stimulate antibody production only when displaying a strongly immunogenic epitope.¹⁸⁴ For example, undecorated β -sheet fibrils and fibrils displaying an RGD motif do not elicit an immune response, whereas fibrils that display a 17-mer sequence from chicken egg albumin stimulate high titer antibody production. These findings suggest that self-assembling peptides may be safe for implantation or injection if strongly immunogenic epitopes are avoided. On the other hand, the ability of β -sheet fibrils to enhance the immunogenicity of selected epitopes indicates that self-assembling peptides may be useful as well-defined adjuvants for vaccine delivery and immunotherapy.

In separate work, Collier and co-workers have demonstrated that, like β -sheet fibrils, undecorated coiled coils do not elicit a measurable immune response.¹⁸⁵ However, triblock materials consisting of coiled-coil endblocks separated by a PEG spacer do stimulate a moderate level of antibody production. They suggest that higher molecular weight oligomers may present better targets to the immune system, a hypothesis that is consistent with the increased immunogenicity of protein aggregates.¹⁸⁶ Although this study was conducted with peptide concentrations below the gel point, it still highlights a key challenge for peptide hydrogels. It may not be possible to predict the immunogenicity of hydrogel networks based on the immune response to precursor peptides and proteins. Further investigation with implanted or injected gels will be required.

5.6.2 New Methods for Peptide Synthesis

While solid-phase peptide synthesis has been used extensively for hydrogel applications, there are several limitations to this method that could hinder efforts to scale up peptide production to clinically and industrially useful quantities. The foremost limitation is the inverse relationship between peptide length and overall yield that precludes the synthesis of polypeptides that contain more than approximately 50 amino acids. Low overall yields and the poor atom economy of reactions involving large protecting groups and coupling reagents also result in significant amounts of wasted starting material. Pattabiraman and Bode have recently reviewed these and other challenges as well as promising new methods of amide bond formation and chemoselective ligation.¹⁸ The development of new reagents and catalysts should be closely followed by the peptide hydrogels field.

Recombinant protein production offers an alternative to SPPS, even for small oligopeptides. Riley *et al.* have recently described the bioproduction of β -sheet peptides that self-assemble into hydrogels.¹⁸⁷ To accomplish this, highly repetitive polypeptides are cleaved into oligopeptides at precise positions using cyanogen bromide. This method should be applicable to nearly any peptide sequence, assuming the appropriate selection of chemical or enzymatic cleavage agents. Like SPPS, there are also drawbacks to recombinant protein

production. Each new recombinant protein requires cloning to produce the template DNA as well as optimization of expression and purification. Protein purification frequently involves an affinity chromatography step and the removal of lipopolysaccharide endotoxins for therapeutic applications. These factors must be considered when choosing between scaling up SPSPS or bio-production of oligopeptides by fermentation.

5.6.3 Spatially Patterned Hydrogels and Epitopes Beyond RGD

Owing to their similar mechanical properties and ability to display bioactive domains, peptide and protein hydrogels are excellent candidates to replace the extracellular matrix (ECM) in tissue engineering scaffolds and *in vitro* cell culture matrices. While the homogeneity and well-defined nature of peptide and protein hydrogels are typically advantageous properties, the natural ECM contains mixtures of physical and chemical signals that vary in each tissue. These signals are often arranged in gradients or spatial patterns that define the cellular microenvironment in such a way as to direct specific cell and tissue behaviors. Hydrogels that mimic these patterns will likely be key to recreating the morphogenic events observed during development and tissue repair.^{8,188} A number of research groups are developing strategies to accomplish this.¹⁸⁹ The West, Shoichet, and Anseth groups have created spatial patterns of cell-binding peptides and protein growth factors within hydrogels using three-dimensional photolithography.^{168,190,191} With these patterns it is possible to direct cell behaviors such as spreading and migration. Straley *et al.* have patterned elastin-like protein hydrogels using spatially controlled enzymatic degradation.¹⁹² Other potential strategies for hydrogel patterning include layer-by-layer assembly using 3D printers^{193,194} and microfluidic approaches.¹⁹⁵

In addition to spatially patterned hydrogels, more realistic cellular micro-environments can be obtained by incorporating biologically active domains beyond the standard RGD cell-binding sequence. While this tripeptide motif is frequently used to demonstrate the cytocompatibility of hydrogels, it binds only a subset of integrin receptors and lacks the spatial context normally provided by neighboring domains in fibronectin, vitronectin, laminin, or collagen.^{196,197} More complex cell-binding domains can be engineered into hydrogels assembled from recombinant proteins. For example, Fong has demonstrated accelerated *in vitro* wound healing on elastin-like protein films containing the full-length fibronectin type III domains 9 and 10.¹⁹⁸ Wound closure on these materials occurs more rapidly than on materials containing only the RGD sequence and approaches the rate observed on fibronectin. It is also possible to include more biological complexity in hydrogels from oligopeptides. Kokkoli and co-workers have developed hydrogels from peptide amphiphiles that contain both the RGD cell-binding domain and the PHRSN synergy sequence separated by the approximate distance observed in fibronectin (H = histidine, N = asparagine).^{199,200} Endothelial cells cultured on these materials exhibit high levels of spreading, extracellular matrix deposition, and cytoskeletal organization. Together, spatial patterning and more complex

epitopes will allow researchers and clinicians to engineer cellular micro-environments that more closely resemble real tissues without compromising the well-defined nature of peptide and protein hydrogels.

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CHAPTER 6

Chemomechanical Hydrogels: Selective Response towards External Effector Molecules

HANS-JÖRG SCHNEIDER

FR Organische Chemie der Universität des Saarlandes,
D-66041 Saarbrücken, Germany
Email: ch12hs@rz.uni-sb.de

6.1 Introduction

Chemoresponsive materials exhibit size changes by interactions with substances in the surrounding medium, representing a special kind of artificial muscle. Hydrogels bearing basic or acidic groups in the polymer backbone undergo volume changes by pH variation. Such gels can be used to detect, for example, glucose after incorporation of glucose oxidase.¹ The application of such enzymes, however, suffers from their tendency to degrade over time; in addition the *in vivo* presence of physiologic buffers attenuates the pH response. Until recently, chemoresponsive hydrogels were mostly triggered by unspecific interactions, *e.g.* by salts or solvents.² Glucose-sensitive hydrogels were already developed in 1992,³ on the basis of covalent interactions with boronic acids (see Section 6.4). In contrast, the chemoresponsive hydrogels mostly dealt with in the present chapter bear binding sites which enable selective non-covalent associations. A related principle is based on hydrogelators, which can exhibit a macroscopic gel-sol transition by external stimuli.⁴ Furthermore, gelation itself can be stimulated or disrupted by competitive guests, including cavitands as additives.⁵

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